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PRINCIPAL INVESTIGATOR: Lewis A. Chodosh, M.D., Ph.D.

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Philadelphia, Pennsylvania 19175

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Chodosh, Lewis A., M.D., Ph.D.

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Philadelphia, Pennsylvania 19104

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Germline mutations in the breast and ovarian cancer susceptibility gene, *BRCA1*, account for a large proportion of families with inherited breast and ovarian cancer. Interestingly, while germline *BRCA1* mutations predispose carriers to adenocarcinoma of the breast, no somatic *BRCA1* mutations have been found in sporadic primary breast cancers. This observation suggests that this molecule may normally protect the breast against carcinogenesis only during specific stages of mammary gland development. Previously, we have analyzed the temporal and spatial pattern of *Brca1* expression during normal mouse embryogenesis, in adult tissues, and during postnatal mammary gland development. These studies support a role for *Brca1* in the regulation of cell proliferation and differentiation in the breast during puberty and pregnancy. We hypothesize that *Brca1* plays a critical role in mammary gland development, and that its function is temporally restricted to particular developmental phases. In this application, we propose to test this hypothesis by using a modified tetracycline-inducible expression system to either induce or abolish *Brca1* expression in transgenic mice during particular developmental stages in a temporally-restricted and breast-specific manner. Through this approach, we hope to understand more clearly how the loss or mutation of this molecule contributes to carcinogenesis in a developmental-specific manner.

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INTRODUCTION

Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5-10% of breast cancer cases result from the inheritance of germline mutations in autosomal dominant susceptibility genes[1, 2]. Germline mutations in one of these breast cancer susceptibility genes, *BRCA1*, appear to account for most families with inherited breast and ovarian cancer, and somewhat less than half of families displaying inherited breast cancer alone[3, 4]. The *BRCA1* gene encodes a 220 kDa phosphoprotein that contains a RING finger motif, a transcriptional activation domain, and a BRCT domain typically found in proteins involved in cell cycle regulation and DNA damage response[4-10]. Tumors arising in patients with germline *BRCA1* mutations almost invariably display loss of the wild-type *BRCA1* allele, suggesting that *BRCA1* is a tumor suppressor gene[11]. Taken together with findings that reduction in *BRCA1* expression *in vitro* results in accelerated growth of breast and ovarian cancer cell lines, and that overexpression of *BRCA1* results in inhibited growth of such cell lines, these observations are consistent with a model in which *BRCA1* negatively regulates proliferation in adult tissues[12-14]. Interestingly, however, we have found that the murine homologue of *BRCA1* is expressed at highest levels in the mouse in cellular compartments containing rapidly proliferating cells undergoing differentiation, such as are found in the breast during puberty and pregnancy[15, 16]. The positive correlation between *Brca1* expression and cellular proliferation may be explained in part by the observation made in several laboratories, including our own, that the expression of this gene is regulated in a cell cycle-dependent manner with peak steady-state levels of mRNA and protein occurring just prior to and during S-phase[5, 17-19]. The discovery that BRCA1 is phosphorylated in a cell cycle-dependent fashion, as well as the finding that *BRCA1* may be a substrate for certain cyclin-dependent kinases, suggests a possible function for *BRCA1* in cell cycle progression and the regulation of proliferation[5, 20]. This hypothesis is supported by reports that *BRCA1* overexpression inhibits cell cycle progression at least in part by upregulating expression of $p21^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor that contributes to the growth arrest response to DNA damage [21, 22]. Interestingly, recent studies have demonstrated that the BRCA1 protein forms a complex with Rad51[23]. Since Rad51 is required for the proper response to ionizing radiation in yeast, these studies suggest a role for BRCA1 in the response to DNA damage. The observation that BRCA1 is rapidly phosphorylated in response to DNA damage, including that caused by ionizing radiation, strongly supports this model[24, 25]. It is important to note, however, that studies of tumor suppressor genes such as *Rb* and *p53* and have highlighted the fact that proteins in this class typically function in multiple pathways and processes in the cell.

Interestingly, while germline *BRCA1* mutations predispose carriers to adenocarcinoma of the breast, no somatic *BRCA1* mutations have been found in sporadic primary breast cancers. This observation suggests that this molecule may normally protect the breast against carcinogenesis only during specific stages of mammary gland development. Previously, we have analyzed the temporal and spatial pattern of *Brca1* expression during normal mouse embryogenesis, in adult tissues, and during postnatal mammary gland development. These studies support a role for *Brca1* in the regulation of cell proliferation and differentiation in the breast during puberty and pregnancy. We hypothesize that *Brca1* plays a critical role in mammary gland development, and that its function is temporally restricted to particular developmental phases. The focus of this project is to test this hypothesis by using a modified tetracycline-inducible expression system to either induce or abolish *Brca1* expression in transgenic mice during particular developmental stages in a temporally-restricted and breast-specific manner. The goal of this work is therefore to understand more clearly how the loss or mutation of this molecule contributes to carcinogenesis in a developmental-specific manner. This goal will be accomplished by pursuing the following specific aims:

Specicic Aim 1. Develop breast-specific, tetracycline-dependent expression systems to inducibly overexpress or abolish *Brca1* expression in the mammary epithelium *in vivo*. We will develop a modified tetracycline-inducible expression system in order to conditionally express *Brca1* or antisense *Brca1* mRNA in the breast in a temporally-restricted manner. Constructs will be generated in which the expression of the reverse tetracycline transcriptional activator, rtTA, will be breast-specific and dependent upon the presence of tetracycline. Since both the expression and function of rtTA are tetracycline-dependent, target expression constructs can be developed in which wild-type or mutant *BRCA1* cDNA clones are expressed in a tetracycline-dependent manner by appending them to *Tn10* operator-containing promoters. Additional target expression constructs will be developed in which *Brca1* antisense RNA is expressed in a tetracycline-dependent manner. Transgenic mouse lines will be generated which overexpress the reverse tetracycline transcriptional activator, rtTA, in a breast-specific manner from the mouse mammary tumor virus LTR. Transgenic mouse lines will also be generated which overexpress the reverse tetracycline transcriptional activator, rtTA, in a tetracycline-dependent manner. Finally, transgenic mouse lines will be generated which contain target DNA constructs that direct the tetracycline-dependent expression of wild-type or mutant *Brca1*, or *Brca1* antisense RNA. These transgenic strains should permit the inducible expression of target transgenes during specific stages of mammary gland development.

Specific Aim 2. Inducibly overexpress *Brca1* in the mammary epithelium of transgenic mice during specific developmental stages. The effect of overexpressing *Brca1* during specific stages of mammary gland development will be determined. Bitransgenic mice which express both the rtTA tetracycline-dependent transcriptional activator and a *Brca1* wild-type or mutant transgene driven by a tetracycline-dependent promoter will be derived by mating each of the transgenic strains developed in specific aim 1. *Brca1* overexpression in bitransgenic mice will be induced during specific stages of mammary gland development, including puberty, pregnancy, lactation and regression, by tetracycline treatment during the appropriate developmental window. Glands will be analyzed by morphological and molecular methods for abnormalities in mammary epithelial proliferation, differentiation and development, and for signs of hyperplasia, dysplasia and neoplasia.

Specific Aim 3. Inducibly abolish *Brca1* expression in the mammary epithelium of transgenic mice during specific developmental stages. The effect of abolishing *Brca1* expression during specific stages of mammary gland development will be determined by creating bitransgenic mice which express both the tetracycline-dependent transcriptional activator, rtTA, and a *Brca1* antisense transgene driven by a tetracycline-dependent promoter by mating the transgenic strains developed in specific aim 1. Reduction of *Brca1* expression in bitransgenic mice will be induced during specific stages of mammary gland development as above. Glands will be analyzed by morphological and molecular methods for abnormalities in mammary epithelial proliferation, differentiation and development, and for signs of hyperplasia, dysplasia and neoplasia.

BODY

Technical Objective I: Develop breast-specific, tetracycline-dependent expression systems to inducibly overexpress or abolish BRCA1 expression in mammary epithelial cells.

***Task 1:** Months 1-12: Construct vectors for expressing rtTA and/or tTA in the mammary epithelium.*

In order to create an inducible expression system in mammary epithelial cells *in vivo*, we have constructed several mammary-specific and tetracycline-dependent expression vectors. Specifically, we have made use of the reverse tetracycline-controlled transcriptional activator, rtTA, that fuses the herpes simplex virus VP16 transcription activation domain with a mutant form of the DNA binding domain from the tet repressor of *E. coli*[26]. This transactivator requires tetracycline derivatives for specific DNA binding. Target genes are placed under the control of the tetO regulatory cassette from the tetracycline-resistance operon of Tn10. This system has been documented to rapidly induce gene expression in the presence of tetracycline by up to three orders of magnitude with a low level of basal expression. Moreover, the availability of numerous tetracycline analogs with varied binding affinities, as well as the use of varying concentrations of tetracycline, permits the absolute level of transgene expression to be reproducibly and precisely titrated. As such, this system is ideally suited for the tight control and rapid induction of potentially toxic genes to desired levels of expression.

Plasmid pUHD172-1neo was constructed by Gossen et al. and contains a neomycin-selectable marker as well as sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by a constitutive CMV promoter/enhancer[26] .

Plasmid pUHD15-1 was also constructed by Gossen et al. and contains a neomycin-selectable marker as well as sequences encoding the tetracycline-controlled transcriptional activator, tTA, whose expression is driven by a constitutive CMV promoter/enhancer[26] .

Plasmid pMMTV.rtTA contains sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by the mouse mammary tumor virus promoter/enhancer and was constructed by replacing the CMV promoter/enhancer of pUHD172-1neo with the entire MMTV promoter/enhancer long terminal repeat (LTR) containing 2.0 kb of upstream sequence. The MMTV LTR is widely used to obtain mammary-specific expression in transgenic mouse model systems.

Plasmid pHMG.rtTA contains sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by the promoter of the housekeeping gene, hydroxymethylglutaryl CoA reductase (HMG CoA). This plasmid was constructed by replacing the MMTV promoter/enhancer of pMMTV.rtTA with the 2.25 kb NotI/XbaI fragment of pHMG. This fragment contains the mouse HMG promoter, the first noncoding exon, and a portion of the first intron. This construct was generated in order to attempt to direct expression to all cells in the mammary gland, inclusive of epithelial cells and stromal cells.

***Task 2:** Months 1-12: Construct vectors for expressing rtTA and/or tTA in a tetracycline-dependent manner.*

Since constitutive expression of rtTA has been reported to be detrimental in some cell types, we have created a modification of the tetracycline-dependent expression system by replacing the constitutive CMV-derived promoter/enhancer driving rtTA expression in pUHD172-1neo, with the tet regulatory sequences (tetO) from Tn10, to create the autoregulatory plasmid, pTetO.rtTA (Fig. 1A). A similar approach has been successfully taken by others with the original tTA tetracycline-repressible transcriptional activator[27]. As a result, in this system the expression of the transcriptional activator, rtTA, is itself induced by the addition of tetracycline, and subsequently induces the target gene in a tetracycline-dependent manner. As a result, induction occurs at two different regulatory levels - the expression of the rtTA activator, and the binding and activation of the target promoter by rtTA.

Task 3: *Months 1-12: Construct target vectors expressing wild type and mutant forms of BRCA1.*

In order to permit the expression of target genes of interest in a tetracycline-dependent manner we have constructed a tetracycline-incucible expression vector, pTet-Target.Puro, that contains both a tetracycline-inducible promoter driving the expression of a target gene, and a puromycin-selectable marker (Fig. 1D). The puromycin-resistance gene, whose expression is driven by the PGK promoter, was obtained from the retroviral vector pLZRS as a ClaI-BspHI fragment, blunted with the Klenow fragment of DNA polymerase, and cloned in the NotI restriction site of pTet-Splice (Life Sciences).

In order to permit the inducible expression of wild-type BRCA1 in mammary epithelial cells in a tetracycline-dependent manner, a full-length cDNA encoding wild-type BRCA1 was subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.wt.

In order to permit the inducible expression of mutant forms of BRCA1 in mammary epithelial cells in a tetracycline-dependent manner, a cDNA clone encoding BRCA1 truncated at the carboxy-terminal ApaI restriction site was subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.ApaI. In addition, a cDNA clone encoding the naturally occurring mutant of BRCA1 C64G was constructed. This mutation contains a point mutation in the RING finger domain of BRCA1 that has been found to cosegregate with breast and ovarian cancer in BRCA1 families. This point mutation was created by PCR site-directed mutagenesis using overlapping PCR primers containing complementary mutational changes at C64. The resulting HindIII-BglI fragment containing the mutant region was subloned into wild-type BRCA1. The full-length HindIII fragment containing the mutant BRCA1 was then subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.C64G.

Task 4: *Months 1-12: Construct target vectors expressing BRCA1 antisense RNA.*

In order to create a system in which *Brca1* expression can be inducibly down-regulated, we have used pTet-Target.Puro to construct a tetracyline-inducible target vector, pTetO-Brca1AS, that inducibly expresses *Brca1* antisense RNA complementary to a 322 nucleotide region at the 5' end of murine *Brca1*. This region spans the putative *Brca1* translation initiation codon. A 322 bp region containing the 5' region of mouse BRCA1 was amplified by RT-PCR from first-strand breast cDNA. This region spans the translation initiation codon of BRCA1. This 322 bp PCR fragment was sequenced on both strands to verify that it contained wild-type sequence and was then subcloned into the HindIII-EcoRV site of pTet-Target.Puro in the antisense orientation to generate pTet.BRCA1.AS. As a negative control, the same 322 bp fragment was subcloned into pTet-Target.Puro in the sense orientation to generate pTet.BRCA1.S. This vector should express the same 322 nucleotide fragment in the sense orientiation.

In order to test the ability of the expression vectors described above to permit tetracycline-dependent inducible expression in mammary epithelial cells *in vivo*, we have used these constructs to generate a test inducible expression system in mammary epithelial cells *in vitro*. We have stably transfected pTetO.rtTA, which contains a neomycin-selectable marker, into HC11 mammary epithelial cells to generate the

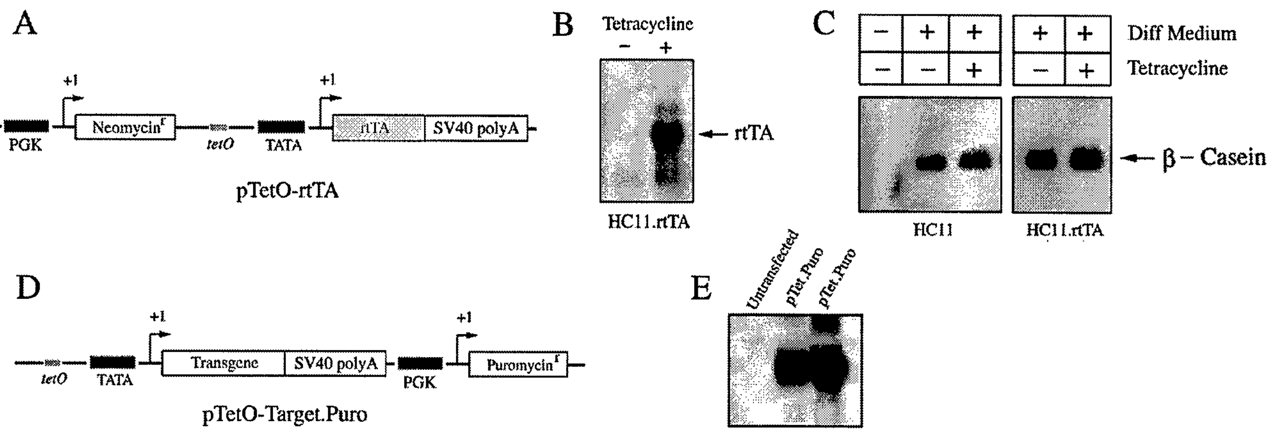

Fig. 1: Generation of a tetracycline-inducible expression system in mammary epithelial cells

mammary epithelial cell clone, HC11.rtTA (Fig. 1A, B). This clone inducibly expresses the rtTA tetracycline-regulated transcriptional activator in the presence of tetracycline (Fig. 1B). In addition, we have demonstrated that the HC11.rtTA clone faithfully differentiates in response to the lactogenic hormones, prolactin, insulin and hydocortisone in the presence or absence of tetracycline, indicating that overexpression of rtTA does not interfere with the process of differentiation (Fig. 1C). Finally, we have demonstrated that induction of HC11.rtTA with doxycycline does not alter proliferation rates in this cell line (not shown). In aggregate, these results demonstrate that the inducible expression of rtTA in mammary epithelial cells represents a suitable system for determining the effect of *Brca1* on mammary epithelial proliferation and differentiation.

In order to test the function of the tetracycline-incucible expression vector, pTet-Target.Puro, that contains both a tetracycline-inducible promoter driving the expression of a target gene, and a puromycin-selectable marker, pTetO-Target.Puro was stably transfected into HC11.rtTA (Fig. 1D). Puromycin-resistant clones obtained following transfection of HC11.rtTA cells with this construct express high levels of mRNA for the puromycin-resistance gene (Fig. 1E). No spontaneously puromycin-resistant clones have been detected following puromycin selection of untransfected HC11.rtTA cells.

In order to test the ability of the tetracycline-dependent transcriptional activator, rtTA, to induce the expression of the tetracycline-dependent target gene in the pTet-Target.Puro expression vector, the parental

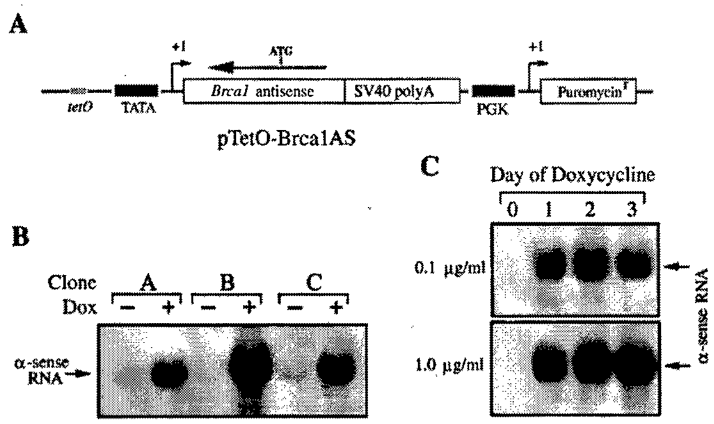

Fig. 2: Graded inducible expression of *Brca2* antisense RNA

HC11.rtTA cell line has been stably transfected with pTet.BRCA1.AS. Puromycin-resistant clones were demonstrated to inducibly express *Brca1* antisense RNA in the presence of doxycycline (Fig. 2B). Induction occurs rapidly, is stable, and occurs in a graded fashion dependent on doxycycline concentration (Fig. 2C). Clones expressing antisense RNA are currently being assayed for their ability to specifically downregulate *Brca1* expression.

Technical Objective II: Create transgenic mice overexpressing BRCA1 or BRCA1 antisense in the mammary epithelium.

Task 1: *Months 8-24: Create and identify transgenic lines of mice expressing rtTA and/or tTA in the mammary epithelium.*

In order to test the hypothesis that *Brca1* plays a role in the normal control of mammary epithelial proliferation and differentiation *in vivo*, *Brca1* will be overexpressed in the mammary glands of transgenic mice. Given concerns regarding possible toxic effects of *Brca1* overexpression, we have chosen to conditionally overexpress *Brca1 in vivo* using a tetracycline regulatory system. We have constructed a mammalian expression vector, pMMTV.rtTA, in which expression of the tetracycline-inducible transactivator, rtTA, is driven by the promoter/enhancer of the MMTV LTR. In order to generate transgenic mice harboring this construct, purified DNA containing the pMMTV-rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated MTA and MTB, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion.

Surprisingly, Northern hybrization analysis of mammary tissue from a large number of MTA transgenic female mice revealed high levels of expression of rtTA in only 50% of male and female animals harboring the MMTV-rtTA transgene. The remaining 50% of transgenic animals had no detectable rtTA expression. Intriguingly, expression of the rtTA transgene in the line of mice has been found to correlate perfectly with the methylation status of the transgene. Animals harboring an MMTV-rtTA transgene with methylation at HpaII sites near the transcription initiation site do not express rtTA in any tissue, wherease animals bearing unmethylated transgenes express rtTA at high levels. Transgenic parents of a given methylation status are able to give rise to progeny that have both methylated or unmethylated transgenes in the same litter. Thus, this phenomenon is not due to a a classical imprinting mechanism. The nature of this effect is currently being investigated further. Regardless, the fact that MMTV-rtTA transgene-positive animals frequently do not express rtTA, makes the MTA line unsuitable for further studies in the inducible expression of BRCA1.

Characterization of rtTA expression in the MTB line was undertaken. Northern hybrization analysis of mammary tissue from four MTB transgenic female mice and two FVB wild-type controls, revealed high levels of expression of rtTA in all four transgenic female animals (Fig. 3). Expression levels were essentially identical in all four animals. As expected, rtTA expression was not affected by treatment of MTB animals with doxycycline. Unlike the MTA founder line, no transgene-positive animals in the MTB line have been detected that do not express the MMTV-rtTA transgene in mammary tissue. This line was therefore selected for further characterization.

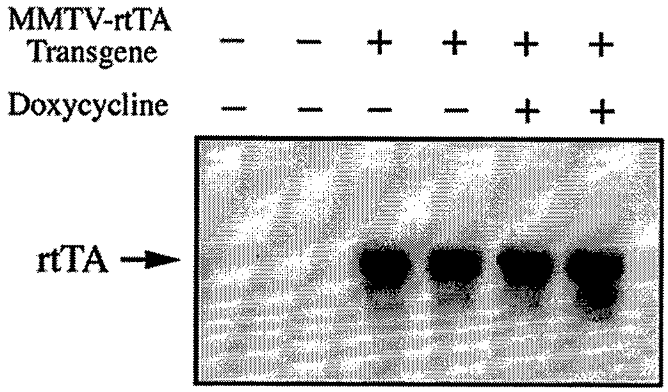

Fig. 3: rtTA expression in MTB transgenic mice

Northern hybridization analysis of 13 tissues derived from male and female progeny of the MTB line of transgenic mice revealed high levels of breast-specific rtTA expression in female virgin animals (Fig. 4). Significant levels of rtTA expression were not detected in other tissues of female mice tested, including brain, skeletal muscle, heart, lung, spleen, kidney, liver, uterus, ovary and testis. rtTA was expressed at low levels in the seminal vesicles of male mice. These results indicated that rtTA is expressed at high levels and in a breast-specific manner in MTB transgenic mice.

We have also constructed mammalian expression vectors, pTetO-LacZ, in which expression of the *LacZ* gene is driven by the tetO-containing promoter cassette, in order to serve as an indicator strain to permit the quantitative characterization of the rtTA/tetO-target bitransgenic expression system created in this proposal. This construct should permit the inducible expression of *LacZ* in response to tetracycline in cells expressing the rtTA transcriptional activator. Using an approach similar to that described above this

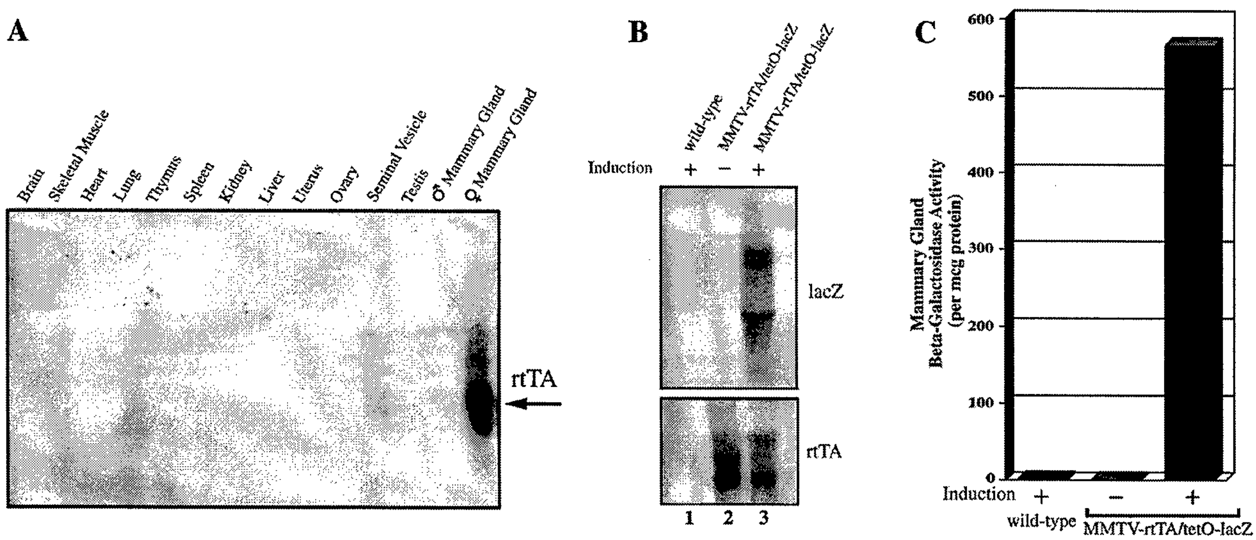

Fig. 4: Bitransgenic system for inducibly overexpressing genes in the breast of transgenic mice

construct, which also contains SV40 splicing and polyadenylation signals, has been used to generate transgenic mice in the FVB background. Founder animals have been identified that pass the pTetO-LacZ transgene to their offspring in a Mendelian fashion.

In order to determine whether this transgenic system will permit inducible transgene expression in the breast in response to tetracycline analogs, MMTV-rtTA transgenic mice were mated to TetO-LacZ mice and bitransgenic mice were identified. Wild-type (non-transgenic) and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. RNA was prepared from these three tissue samples and steady-state levels of *rtTA* and *LacZ* mRNA expression were assessed by Northern hybridization (Fig. 4B). As expected, bitransgenic animals expressed *rtTA* at similar levels in the presence and absence of doxycycline, whereas the *LacZ* target mRNA was only expressed in bitransgenic animals treated with doxycycline. No *LacZ* mRNA was detected either in wild-type animals, or in untreated bitransgenic animals.

In order to quantitate the level of lacZ protein expression in the induced and uninduced states, beta-galactosidase assays were performed on protein extracts made from each of the harvested breast tissues (Fig. 4C). As expected, no beta-galactosidase activity was detected in extracts prepared from non-transgenic breast tissue. Also as expected, abundant beta-galactosidase activity was present in extracts prepared from bitransgenic animals treated with doxycycline for 48 hours. Impressively, no beta-galactosidase activity was detected in protein extracts prepared from untreated bitransgenic animals. Based on the lower limits of detection for this assay, we estimate that the minimum induction of transgene expression observed in this experiment is at least 700-fold, and that the actual level of induction may be 1 or 2 logs higher. Our estimates of the minimum level of induction are comparable to that observed in a related system making use of the tTA tetracycline-dependent repressor, rather than the rtTA tetracycline-dependent transactivator[28].

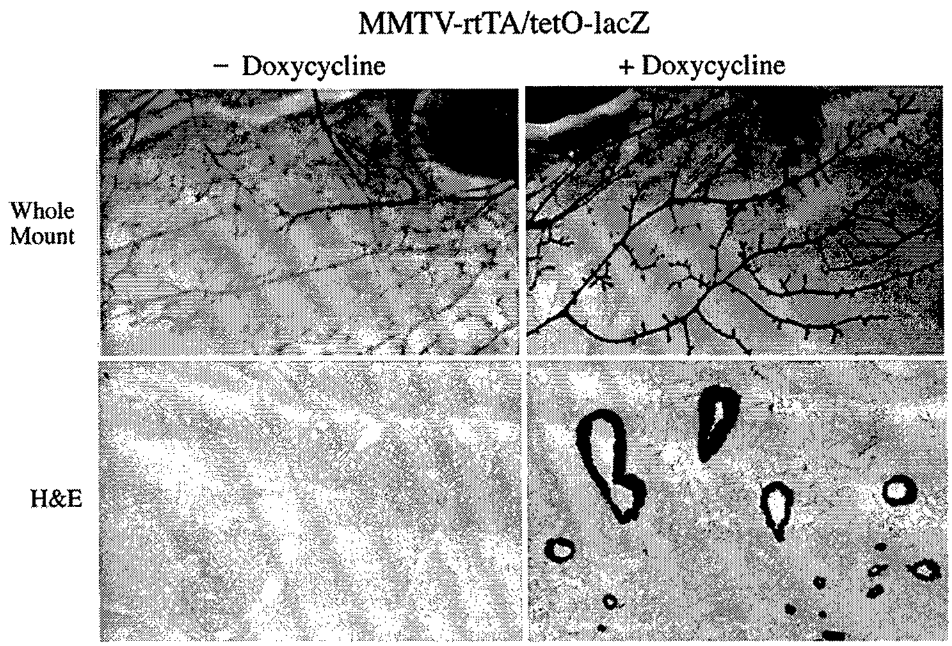

Fig. 5: LacZ expression in the mammary epithelium of bitransgenic mice

In order to confirm these results and to identify the cell types in the mammary gland in which inducible expression occurred, MMTV-rtTA transgenic mice were mated to TetO-LacZ mice and bitransgenic mice were identified. Bitransgenic mice were treated with doxycycline and breast tissue was harvested after 48 hours, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Both mammary gland whole-mounts and frozen sections from mammary glands embedded in OCT were stained *in situ* for lacZ activity (Fig. 5). Results from both whole-mounts and frozen sections demonstrated that inducible lacZ expression is confined to the mammary epithelial tree. Moreover, expression occurred at a high level and in a relatively homogeneous manner in all epithelial structures examined.

In aggregate, our results demonstrate that the MMTV-rtTA/TetO-transgene system that we have generated will permit the rapid and breast-specific induction of transgene expression at high levels, in combination with extremely low levels of expression in the uninduced state. Notably, this system permits the modulation of transgene expression levels both by varying the concentration of doxycycline and by varying the tetracycline derivative used. These properties are ideal for determining the effects of a potentially toxic transgene on specific stages of mammary gland development, and for obtaining levels of transgene expression that are in the physiological range.

In order to create transgenic lines of mice that express rtTA constitutively in all cell types in the mammary gland, including epithelial cells and stromal cells, the pHMG.rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated HTA and HTB, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion. In order to determine whether this transgenic system will permit inducible transgene expression in the breast in response to tetracycline analogs, HTA and HTB transgenic mice were mated to TetO-LacZ mice and bitransgenic mice were identified. Wild-type (non-transgenic) and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Beta-galactosidase assays were performed on protein extracts prepared from each of these harvested breast tissues. No beta-galactosidase activity was detected in bitrasngenic HMG.rtTA/tetO-LacZ mammary tissue from animals induced with doxycycline. We conclude that the HTA and HTB lines of mice will not be suitable for expressing inducibly target transgenes in the mammary gland.

In order to create transgenic lines of mice that express rtTA in a tetracycline-dependent manner, purified DNA containing the pTetO.rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated TTB and TTE, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion. Northern hybridization analysis of tissues derived from progeny of the TTB and TTE lines of mice failed to reveal detectable levels of rtTA expression in the mammary glands of female virgin animals (not shown). Low levels of expression of rtTA were detected in TTB and TTE animals in the thymus, kidney and spleen. In order to confirm these results, the TetO.rtTA transgenic lines of mice, TTB and TTE, were mated to TetO-LacZ mice and bitransgenic tetO.rtTA/tetO-LacZ mice were identified. Wild-type and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Beta-galactosidase assays were performed on protein extracts prepared from each of these harvested breast tissues. No beta-galactosidase activity was detected in bitrasngenic tetO.rtTA/tetO-LacZ mammary tissue from animals induced with doxycycline. We conclude that the TTB and TTE lines of mice will not be suitable for inducibly expressing target transgenes in the mammary gland.

***Task 2:** Months 8-24: Create and identify transgenic lines of mice expressing wild type and mutant forms of BRCA1 in a tetracycline-dependent manner.*

As described above, we have constructed a mammalian expression vector, pTetO.BRCA1, in which expression of *BRCA1* is driven by the tetO-containing promoter cassette. This constructs should permit the inducible expression of *BRCA1* in response to tetracycline in cells expressing the rtTA transcriptional activator. Using an approach similar to that described above this construct, which also contains SV40 splicing and polyadenylation signals, has been used to generate transgenic mice in the FVB background. To date we have identified two founder animals, TB1A and TB1B, that pass the pTetO-BRCA1 transgene to its offspring in a Mendelian fashion. In addition, in order to generate more founder lines in order to increase the likelihood of identifying a tetO-BRCA1 line of transgenic animals that is able to inducibly express BRCA1 in response to doxycycline, we have reinjected purified pTetO.BRCA1 DNA into fetilized oocytes from superovulated FVB mice. This has resulted in the generation of an additional 7 founder animals that contain this transgene in their tail-derived DNA. These additional lines of mice are being bred to wild-type FVB mice in order to determine whether they pass this transgene to their offspring in a Mendelian fashion.

***Task 3:** Months 8-24: Create and identify transgenic lines of mice expressing BRCA1 antisense RNA in a tetracycline-dependent manner.*

Before creating transgenic mice expressing *BRCA1* antisense RNA in a tetracycline-dependent manner, we wished to determine whether the pTetO-BRCA1.AS construct generated above would be capable of down-regulating *BRCA1* expression. In order to accomplish this, the antisense constructs are first being tested for their ability to specifically reduce the expression of BRCA1 protein using the HC11 *in vitro* test system that we have generated. Achieving this goal requires the specific detection of mouse Brca1 protein. We have tested several commercially available antisera directed against mouse Brca1. In our hands, none of these antisera is capable of specifically detecting mouse Brca1. As a result, we have chosen to generate our own anti-mouse Brca1 antisera. GST fusion proteins from four regions of the mouse Brca1 protein have been generated and expressed in E. coli. GST fusion proteins will be cleaved, purified and each will be injected into two rabbits. The resulting antisera will be tested for their ability to specifically recognize the mouse Brca1 protein. Once this goal has been accomplished, we will screen the above HC11 clones that inducibly express BRCA1 antisense in order to determine whether the antisense fragments selected are capable of down-regulating Brca1 at the protein level.

If these experiments are successful, a DNA fragment containing the pTetO-BRCA1.AS expression cassette will be injected into fertilized oocytes harvested from superovulated FVB female mice, using an approach similar to that described above. Founder mice will be identified that harbor the transgene in tail-derived DNA and that pass this transgene to offspring in a Mendelian fashion.

***Task 4:** Months 8-24: Breed transgenic lines of mice to create a bitransgenic line of mice expressing wild type and mutant forms of BRCA1 in the breast in a tetracycline-dependent manner.*

MMTV-rtTA, MTB, transgenic mice identified above have been mated to offspring of the TetO-BRCA1, TB1A and TB1B, transgenic line described above. Bitransgenic offspring have been identified by PCR. The expression of BRCA1 has been induced in bitransgenic animals by treatment with doxycycline for 48 hours. Breast tissue has been harvested from treated and untreated bitransgenic animals as well as control non-transgenic animals. BRCA1 expression in the breast has been assayed by Northern hybridization. No BRCA1 mRNA was detected by this means, however, endogenous BRCA1 mRNA cannot be detected in the breast by Northern hybridization either. In order to perform a more sensitive assay for BRCA1 expression, we will perform RNase protection assays at the mRNA level, and immunoblotting at the protein level. In addition, the seven additional founder animals harboring the pTetO-BRCA1 will also be characterized by this approach if they are demonstrated to pass the TetO-BRCA1 transgene to their offspring in a Mendelian fashion.

Task 5: *Months 8-24: Breed transgenic lines of mice to create a bitransgenic line of mice expressing BRCA1 antisense in the breast in a tetracycline-dependent manner.*

The completion of this task is pending at the current time. Completion of this task will depend on the successful completion of Task 3 in which transgenic lines of mice are created that express BRCA1 antisense RNA in a tetracycline-dependent manner, and that downregulate the Brca1 protein when tested in HC11 cells..

Technical Objective III: Determine the effect of inducibly overexpressing BRCA1 in the mammary epithelium of transgenic mice during specific developmental stages.

Task 1: *Months 24-48: Analyze the phenotype of inducibly overexpressing wild type BRCA1 in the mammary epithelium during specific developmental stages.*

The completion of this task will depend on the successful completion of tasks described above in which bitransgenic lines of mice are generated that express wild type forms of BRCA1 in the breast in a tetracycline-dependent manner.

Task 2: *Months 24-48: Analyze the phenotype of inducibly overexpressing mutant forms of BRCA1 in the mammary epithelium during specific developmental stages.*

The completion of this task will depend on the successful completion of tasks described above in which bitransgenic lines of mice are generated that express mutant forms of BRCA1 in the breast in a tetracycline-dependent manner.

Technical Objective IV: Determine the effect of inducibly abolishing BRCA1 expression in the mammary epithelium of transgenic mice during specific developmental stages.

Task 1: *Months 24-48: Analyze the phenotype of inducibly abolishing BRCA1 expression in the mammary epithelium during specific developmental stages.*

The completion of this task will depend on the successful completion of tasks described above in which bitransgenic lines of mice are generated that express BRCA1 antisense RNA in the breast in a tetracycline-dependent manner and that result in the specific reduction in BRCA1 protein in mammary epithelial cells.

CONCLUSIONS

A number of important milestones have been accomplished during the first two years of this project. We have constructed a variety of plasmid vectors for expressing rtTA in the mammary epithelium in a tetracycline-dependent or tetracycline-independent manner. We have also created target vectors for expressing wild-type and mutant forms of BRCA1, as well as target vectors for expressing BRCA1 antisense RNA. These vectors have been used to create four transgenic lines of mice containing the TetO-rtTA, MMTV-rtTA, TetO-LacZ and TetO-BRCA1 expression cassettes. Experiments to date demonstrate that the MMTV-rtTA transgenic line of mice express the tetracycline-dependent reverse transcriptional activator, rtTA, at high levels, in a breast-specific manner, and in a mammary epithelial-specific manner. The creation of bitransgenic MMTV-rtTA/TetO-LacZ mice has permitted the initial analysis of the utility of this inducible transgenic system. These results strongly suggest that this system is capable of inducing target gene expression to high levels in a breast-specific fashion in response to induction with tetracycline derivatives. In addition, this system demonstrates extremely low levels of basal expression.

The studies described above demonstrate that we have made significant progress towards the completion of the specific aims of this project during the first two years. All technical objectives that were predicted to be complete by the end of the first year of this project have been successfully comleted. Some of the technical objectives that were predicted to be comlete by the end of the second year have been successfully completed, and all of these objectives are well on their way towards completion. These findings suggest that the experiments proposed can be executed in a timely fashion to answer important scientific questions regarding the function of the breast cancer susceptibility gene, BRCA1, in the mammary gland.

Publications during the current contract year:

Chodosh, LA. *BRCA1* and *BRCA2* expression in normal and neoplastic cells. *Journal of Mammary Gland Biology and Neoplasia* 1998 (In Press).

Chodosh LA, D'Cruz CM, Gardner HP, Ha SI, Marquis ST, Rajan JV, Stairs DB, Wang JY, and Wang M. Mammary gland development, reproductive history and breast cancer risk. *Cancer Research* 1998 (In Press).

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

Rec'd 7/23/2001

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FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management